IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 4, Issue 2 (Mar. - Apr 2018), PP 78-81
www.iosrjournals.org

Molecular Characterization and Alchohol Tolerance of *Saccharomyces Cerevisiae* Used In Bioethanol Production From Waste Paper

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Abstract: *Sacchaomyces cerevisiea has been widely used in the production of bioethanol from several substrates. This study portrays the molecular characterization and alcohol tolerance of Sacchaomyces cerevisiea used in the production of bioethanol from waste paper. The Sacchaomyces cerevisiea isolate used with ascension number MG584866 was shown to have 99% similarity to its gene bank relative Sacchaomyces cerevisiea with ascension number CP006454. The alcohol tolerance of the Sacchaomyces cerevisiea recorded 100% mortality at 40% alcohol concentration, while 0% mortality was recorded at 0% concentration. The isolate was however able to survive 30% alcohol concentration where 76% mortality was recorded.*

Date of Submission: 26-03-2019 Date of acceptance: 09-04-2018

I. Introduction

Despite efforts to search for new microorganisms, the yeast *Saccharomyces cerevisiae* remains the most utilized for ethanol production in Nigeria. It is a robust yeast that is capable of withstanding stressful conditions and has a high fermentation efficiency, rapid growth, effective sugar use, the ability to produce and consume ethanol, tolerance of high ethanol concentrations and low levels of oxygen, osmotolerance, thermotolerance, and cell activity in acidic environments, which are fundamental to its industrial usefulness (Andrietta et al., 2007).

Degradation of cellulose is the breakdown of cellulose into glucose subunits. Microbiologically, cellulose is mainly degraded by an enzyme known as cellulase which is commonly produced by cellulolytic bacteria and fungi e.g *Saccharomyces cerevisiae, Aspergillus sp,Pleurotus ostreatus* (edible mushroom) e.t.c.

Studies have revealed that white paper has abundance of cellulose, hence its choice for the production of bioethanol. Currently, bioethanol production is focused on sugar crops including sugar cane, sugar beets and starch crops, including wheat, potatoes and sweet potatoes, which is often based on excess agricultural production and it is generally recognized that this volume is too small in comparison with the anticipated levels of production required for total conversion of transportation fuel markets from gasoline to ethanol. Not only is it renewable, these biofuels can also reduce emission of gases which potentially can cause global warming (Stanley., 2010).

II. Materials And Methods

Source of samples

Palm wine sample for *Saccharomyces cerevisiae* isolation was purchased from local market in Alimini Community in Emouha L.G.A of Rivers State, and transported to the laboratory within 12hrs of collection.

Isolation/identification of *Saccharomyces cerevisiae* from palm wine

A 10-fold serial dilution of 1ml sample of the palm wine was made, and 1ml plated out on PDA media in duplicates. This was incubated at 30^0C for 24 hrs and sub cultured to get the pure culture of the isolate.
Macroscopic and microscopic identification of *Saccharomyces cerevisiae* were done and recorded according to Larone's manual (1995), after which the molecular identification of the isolate was done.

Molecular Identification

DNA extraction (Boiling method)

Five milliliters of an overnight broth culture of the yeast isolate in Luria Bertani (LB) was spun at 14000rpm for 3 min. The cells were re-suspended in 500ul of normal saline and heated at 95^0C for 20 min. The heated fungal suspension was cooled on ice and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml micro centrifuge tube and stored at -20^{o}C for other down stream reactions.

DNA quantification
The extracted genomic DNA was quantified using the Nano-drop 1000 spectrophotometer

16S rRNA Amplification
The 16s RRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator.

Sequencing
Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa.

Phylogenetic Analysis
Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbour-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

Alchohol Tolerance Test of *Saccharomyces cerevisiae*
Preparation of test medium
The different concentrations (0, 5, 10, 15, 20, 25, 30mg/l) of ethanol was prepared using potato dextrose broth medium and dispensed into different test tubes labeled appropriately.

Preparation of test organisms
Loopful of the test organisms (*Aspergillus niger* and *Saccharomyces cerevisiae*) were transferred into 10ml sterile potato dextrose broth and incubated for 2days at room temperature. 1ml of aliquot of each culture were transferred into fresh sterile broth and incubated for 24 hours to ensure that actively growing organisms were used for toxicity test.

Alcohol tolerance test procedure
Tubes containing 9ml of the different ethanol concentrations (0, 5, 10, 15, 20, 25, 30mg/l) of potato dextrose were set up. 1ml of the test organisms from the actively growing broth were added to their respective labeled tubes and incubated for 48hrs at room temperature. 0.1ml from each tube was plated out potato dextrose agar plates and incubated for 48hrs at room temperature. The colonies were then counted and average colony taken.

The percentage log survival of isolates in the toxicants
The percentage log survival of the isolates in the alcohol concentration used in the study was calculated using the formula adopted from Williamson and Johnson (1981). This was calculated by obtaining the log of the count in each alcohol concentration and dividing the count in the zero alcohol concentration and then multiplying the product by 100.
Therefore %log survival = $\frac{Log\ C}{Log\ c} \times 100$

Where $Log\ C$ = log count in each alcohol concentration,
$Log\ c$ = log count in the zero alcohol concentration.

III. Results

The Microscopic and Macroscopic characteristics of *Saccharomyces cerevisiae* are represented in Table 1. In order to isolate *S. cerevisiae* strains without bacterial contamination the samples were submitted to a set of sequential media supplemented with antibacterial substances.

Table 1 Microscopic and Macroscopic identification of *Aspergillus niger*, and *Saccharomyces cerevisiae*

Macroscopy	Microscopy	Probable genera
Cream, smooth, glabrous yeast-like colonies.	Large globose to ellipsoidal budding yeast-like cells	*Saccharomyces cerevisiae*

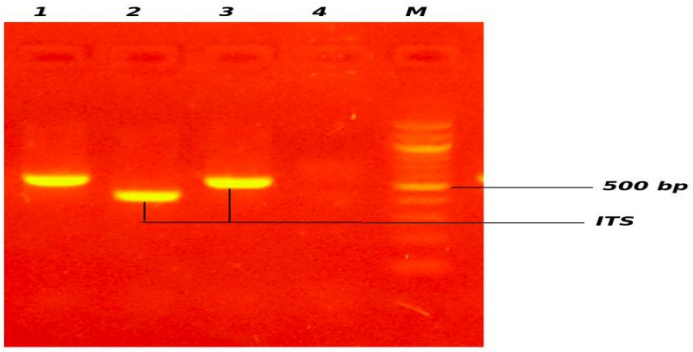

Fig 1: Agarose gel electrophoresis showing the amplified ITS bands of the fungal isolates. Lanes 1, 2, 3 represent the ITS bands, lane 4 represents the negative control wile Lane M represents the 100 bp molecular ladder.

Table 2: Fungal isolates used for cellulose degradation and fermentation

Code	Organism	Accession number	GenBank relative	Accession number	Percentage similarity
F5	***Saccharomyces cerevisiae***	**MG584866**	***Saccharomyces cerevisiae***	**CP006454.1**	**99**

The phenotypic and tentative identities of the fungi are presented in table 1. The table shows the representative genera of the isolate from palm wine sample. The yeast genera isolated from palm wine sample was identified as *Saccharomyces* spp. Bands from amplified PCR products as shown in fig. 1 were purified and sequenced. Identification of yeast sequence was aligned with BLAST search facility of National Center for Biotechnology Information (NCBI) database. The sequence aligned gave 99% similarity as presented in table 2. Sequence result obtained was *Saccharomyces cerevisiae* with ascension number MG584866, whose gene bank relative was *Saccharomyces cerevisiae* with ascension number CP006454.1.

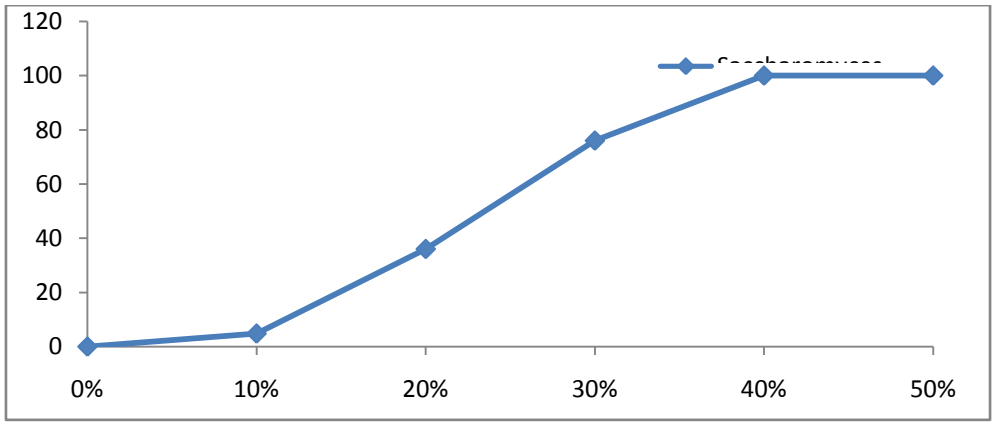

Fig 2. Alcohol Tolerance of *Saccharomyces cerevisiae*

Figure 2 is a graphical representation of alcohol Tolerance on *Saccharomyces cerevisiae.* The results portrayed 100% mortality for the test organism at 40 to 50%, while 30% concentration expressed mortality of 76%. Mortality of 4.8 and 36% were recorded for 10 and 20% concentration respectively. The control (0% concentration) recorded 100% survival of the test organism.

IV. Discussion And Conclusion

The microscopic and macroscopic characteristics of yeast isolated was similar the one isolated by Adeyemo and Sani (2013**).** The isolate was further identified using molecular techniques, and after aligning the sequence with BLAST search facility of National Center for Biotechnology and Information (NCBI) database, the result showed 99% similarity with *Saccharomyces cerevisiae* with ascension number CP006454.1. The molecular result is presented in table 2.

The alcohol tolerance of *Saccharomyces cerevisiae* was however tested to determine the concentration of alcohol that will be able to inhibit the growth of the fermenting organism (*Saccharomyces cerevisiae*). The result of the percentage survival of the test organism with increasing concentration is represented in fig 2. The result demonstrates that concentration of 40% was lethal to the test organism in that it was able to kill 100% of the test organism. This result contradicts with reports of Ghareib *et al*., 2008 who reported that 14% ethanol was completely inhibitory to *Saccharomyces cerevisiae.*

In conclusion, the present study allowed the isolation, and molecular characterization of *Saccharomyces cerevisiae*, and the alcohol tolerance of the isolate. This organism could be very useful in production of bioethanol from waste paper.

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IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB) is UGC approved Journal with Sl. No. 4033, Journal no. 44202.

A.O. Erewa. " Molecular Characterization And Alchohol Tolerance Of Saccharomyces Cerevisiae Used In Bioethanol Production From Waste Paper." IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB) 4.2 (2018): 78-81.